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(54) Title: TISSUE CULTURING

(57) Abstract

Controlled release nutrients are added to tissue culturing environments to replace and supplement essential nutrients consumed by the growing tissue.

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TISSUE CULTURING

BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates to an improvement in tissue culturing. More particularly, it relates to an improved method for delivering biologically active materials such as nutrients to tissue culture environments.

Background Information

"Tissue culturing" is a general term applied to micropropagation processes in which tissues or cells of higher animals or plants are grown artificially in a controlled environment. In these processes, tissues or cells, either as suspensions or as solids are maintained under conditions conducive for their growth and multiplication. These conditions include proper temperature, proper gaseous and liquid environment, and proper supply of nutrient.

Tissue culturing, as applied to plants, is presently viewed as an expensive method. Although

micropropagation represents one of the few means by which many forestry, plantation and other difficult-to-root species can be clonally reproduced, the high cost of tissue culture techniques has prevented broader application in the marketplace. Consequently, the appearance of clonal forests, fields and crops has not materialized.

A great opportunity exists for the micropropagation industry. However, this opportunity is contingent upon driving down the cost of micropropagated plantlets below 10 cents per unit. Several components contribute to the high cost of the process. First, the process is labor intensive because of repetitive manual operations as they are currently carried out. Labor accounts for 38-45% of the final cost of each plantlet, or 60% of the direct costs. (Donnan, A., Jr., (1986) "Determining and Minimizing Production Costs" in Zimmerman, R.H., et al. (eds.) Tissue Culture as a Plant Production System for Horticultural Crops, pp. 167-174.) Second, the facilities costs add to the generally high laboratory overhead. Third, the requirement for sterility contributes to the plantlet price both directly, by limiting the size of the culture vessel, and indirectly, as a function of the impediment it presents to automation and to ease of handling.

The primary reason for the labor intensity and the concerns about sterility resides in the need to supply nutrients and medium salts to the growing tissues or cells. The influence of medium salts on plantlet quality is significant. Many plants, especially woody species, have been shown to deplete the nutrients and salts in their medium during the first two weeks of subculture (Mezzetti, B., et al., Actinidia Chinensis, C.V. Hayward, Nutrition in Proliferation. Abstracts: Tissue Culture as a Plant Production System for Horticultural Crops. October 20-23, 1985, Beltsville, Maryland. Abstract No. 22). More frequent transferring is impractical from a

commercial perspective, and increasing the initial salt and nutrient concentration is limited by plantlet tolerance to salinity.

An alternative to this frequent transferring is to devise a system in which a continuous flow of medium is passed over the growing tissue. This has been demonstrated on a laboratory basis and works well. However, the flow-through containers can have problems with sterility and are very complicated and expensive. In a field where cost reduction is a major desired end, the use of more complicated, more expensive equipment is generally to be avoided. See, for example, U.S. patent 4,537,860 of Tolbert et al. for a typical static maintenance system. European patent application 132,414 shows a complicated automated system useful in plant tissue culturing.

The present invention seeks to address these problems and provide a cost effective way for delivering nutrients and/or essential minerals or other needed components to a tissue culture environment.

STATEMENT OF THE INVENTION

It has now been found that controlled release technology, a technology primarily employed in drug delivery, pesticide delivery, and fertilizer delivery to potted plants and field crops, can advantageously maintain levels of nutrients, minerals, and other biologically active essential growth elements in a tissue culture medium at levels at or near optimum for sustained periods.

Thus, in one aspect this invention provides an improved tissue-culturing process in which plant cells are grown in an aqueous-based gel matrix containing nutrients essential to the growth of the cells. In this process the improvement involves placing in that growth medium an

insoluble or slowly soluble depot of one or more of the nutrients or other biologically active materials. The depot has the property of being able to continuously release the one or more agents it contains into said growth medium over a prolonged period of time, thereby replacing the agents consumed by the growing cells and maintaining the agent level at or near optimum levels.

The present invention finds principal application with plant tissue cultures. In the case of plant materials, the nutrients which are delivered can include salts, organic growth regulators, as well as sugars and other materials consumed by the growing plant.

The depots employed are generally made up of one or more bodies of nutrient and/or agent surrounded by a coating which limits the rate of dissolution or release of the body of nutrient or agent into the medium.

In a preferred embodiment, the sustained release nutrient sources are used in combination with an initial loading of wholly soluble nutrients and agents. The initial loading serves to provide levels of nutrients and agents adequate for initial growth, and the sustained-release material adds nutrient and agents as they are consumed by the growing tissue.

DETAILED DESCRIPTION OF THE INVENTION

Brief Description of the Drawing

This invention will be described with reference being made to the accompanying drawings, in which

Figure 1 is two graphs showing the changes in conductivity observed over a 6 week period in agar (A) and water (B) tissue culture media when six different levels of controlled release nutrients are added.

Figure 2 is a graph similar to Figure 1 showing the release of nutrient into a tissue culture medium

containing an initial concentration of Murashige-Skoog's salts.

Figure 3 is a series of graphs showing the depletion of nutrient from a tissue culture environment when various plants are cultured.

Figure 4 is a series of graphs showing the sum of the depletion shown in Figure 3 plus the release of material released by the controlled release nutrient.

Figure 5 is a graph comparing the cumulative release of controlled release nutrient into a tissue culture medium with the cumulative uptake of nutrient by three species of plants in a tissue culture.

Figure 6 is a graph showing relative growth observed with Ficus benjamina when various levels of controlled release nutrient are added.

Figure 7 is a graph showing the relative biomass of Boston fern observed when various levels of controlled release nutrient are added.

Figure 8 is a graph showing the relative growth of potatoes observed when various levels of controlled release nutrient are added.

Description of Preferred Embodiments

This Description of Preferred Embodiments contains the following sections:

Typical Tissue Culture Environments
Agents Typically Delivered by the Invention
Controlled Release Systems
Experimental Results

Typical Tissue Culture Environments

The typical environments in which the present invention are employed comprise an aqueous gel medium in which cells or living tissue are artificially grown. Typically, these environments are small separately enclosed sterile regions such as closable jars, culture

dishes and the like. The environment includes an aqueous growth medium. This is, in the case of plants, typically an agar-based medium which contains substantial quantities of sugars such as sucrose, glucose, and coconut milk and essential minerals and the like. The environment also includes needed gases such as CO_2 or oxygen and, in the case of plants, a source of light. United States Patent 3,819,960 is incorporated herein by reference and includes a description of the various materials including minerals, metal salts, growth hormones, amino acids and vitamins which may be advantageously present in the growth environment. United States Patent 4,038,778, also incorporated by reference, shows other culturing conditions.

Agents Typically Delivered by the Invention

The agents delivered to the growth environment using the present invention, in the case of plant tissue cultures, are most typically water-soluble macronutrients such as potassium salts, nitrogen containing salts, and phosphorous containing salts. (As will be apparent, the term "nutrient" is used expansively to include macronutrients, micronutrients and growth factors, vitamins, hormones and the like.) These materials are consumed at a rapid rate during plant growth or subculture but if present at any point in too high a concentration can lead to toxicity in the growth environment. Micronutrients such as salts or other soluble compounds of iron, zinc, manganese, copper, nickel, molybdenum and boron can also be added. Organic materials can also be added including vitamins, growth factors and growth regulators and the like.

The invention could be used to deliver all the macro and micronutrients to a tissue-culturing medium. This would have the potential disadvantage of requiring an initial induction period when the nutrient slowly releases and the nutrients build up to a proper level.

More typically, the environment is started with an initial loading of macro and micronutrients. The controlled release material is then used to replace nutrients as they are consumed and maintain nutrient levels at or near optimum levels.

Thus, the invention can be used in combination with known nutrient sources at or below their usual levels.

A typical known plant nutrient package is Murashige-Skoog's salts which are typically used at from 0.4 to 1.0 and preferably 0.4 to 0.8 times its normal use levels. (See Revue Physiologia Plantarum 15:473 for details on Murashige-Skoog's salts.)

In preferred embodiments, a typical salt mixture is used to supply macro and micronutrients and the controlled release material is used to supply macronutrients as they are consumed by the growing plants.

Representative nutrients in tissue culture media include the following.

Macronutrients: potassium and ammonium nitrates, magnesium sulfate, calcium chloride, and monopotassium phosphate (Murashige-Skoog's macroelements).

Micronutrients include manganese, zinc and copper sulfates, potassium iodide, boric acid, calcium chloride and molybdenum-sodium oxide (Murashige-Skoog's microelements).

In a variation, the calcium chloride and molybdenum-sodium oxide is replaced by aluminum chloride and nickel chloride to give a mixture known as "Heller micronutrients" and described in Culturing Plant Tissues by R. Gaulherst, 1959, Ed Masson and Co.

Typical levels which one would use with 1.0 times M/S salts are follows:

Macroelements	Wt in mg/l of Medium
NH_4NO_3	1650
KNO_3	1900
$CaCl_2(2H_2O)$	440
$MgSO_4(7H_2O)$	370
KH_2PO_4	170

Microelements	Wt in mg/l of Medium
$MnSO_4(4H_2O)$	22.3
$ZnSO_4(7H_2O)$	8.6
H_2BO_3	6.2
KI	0.830
$Na_2MoO_4(2H_2O)$	0.350
$CuSO_4(5H_2O)$	0.025
$CaCl_2(6H_2O)$	0.025

or in the Heller system

Microelement	Use Level (mg/l)
H_2PO_3	1.0
$ZnSO_4(7H_2O)$	1.0
$MnSO_4(H_2O)$	0.076
$CuSO_4(5H_2O)$	0.030
$AlCl_3(6H_2O)$	0.050
KI	0.010
$NiCl_2(6H_2O)$	0.030

Iron may also be present such as at 27.3 mg/l of $FeSO_4(7H_2O)$. Cobalt is another typical constituent such as at 0.02 mg/l of $CoCl_2(2H_2O)$.

Typical vitamins and growth factors can include:

Meso-inositol	100	mg/l
Calcium pantothenate	1.0	mg/l
Nicotinic acid	1.0	mg/l
Vitamin B_6	1.0	mg/l
Vitamin B_1	1.0	mg/l
Biotin	0.01	mg/l
Thiamine	0.04	mg/l

Growth regulators such as naphthaleneacetic acid or benzyladenine can be present if desired.

In preferred embodiments, the initial culturing media will contain these microelements and up to these levels of macroelements with the controlled release material replenishing macroelements and optionally microelements as they are consumed.

Thus, in one embodiment, the invention can employ a controlled release material capable of delivering and maintaining the appropriate levels of N, P and K.

In another embodiment, the controlled release material can deliver N, P, K, Ca and Mg.

In yet another embodiment, the controlled release material can deliver N, P, K, Ca and Mg and at least one of Cu, Co, Mo, I, Zn, B, Mn, and Fe.

In the case of animal tissue culturing systems, the present invention can be used to deliver vitamins, hormones, essential nutrients and the like.

Controlled Release Systems

In accord with the present invention, nutrients are delivered to tissue culturing environments by the use of controlled release systems. These systems are made up of a depot of nutrient and means to prevent the immediate

release of the nutrient into the tissue culture environment of use. Typically, the nutrients are water soluble solids or, at times, liquids. As already noted, the environment of use is aqueous.

Any release mechanism which will prolong the release of the nutrients over a period of not less than 7 days can be used, with preferred systems prolonging and extending the release period to between 7 and 360 days and especially between 21 and 150 days.

In selecting a release mechanism it should be kept in mind that the degree of precision required is not extreme and that in most cases one can safely trade off precise control of release kinetics for cost savings. As will be obvious, in the areas of commercial plant production, cost control is of utmost importance.

One useful and often cost effective release mechanism is to disperse the particles of nutrient in a solid body of slowly soluble material such as a slowly soluble polymer. Slowly degradable esters such as the orthoesters or slowly hydrolyzable materials can be used as an erodible matrix.

Another release mechanism which can be employed involves the use of a semipermeable layer surrounding one or a plurality of depots of the nutrients which layer permits the passage of water into the depot with the subsequent gradual dissolution of the soluble salts (nutrients) and thus the generation of osmotic pressure within the depots. This osmotic pressure can be used to drive the nutrients out of the depots either through preprovided microholes or through holes formed in situ by the pressure build up. A seemingly limitless family of materials are available to use as these osmotic membranes and include cellulose acetate, various resins and the like. A representative listing of typical osmotic membrane materials is included in United States Patent 3,845,770, which is incorporated herein by reference.

Other representative disclosures of controlled release coatings can be found in United States Patents 4,019,890; 3,342,577; 4,369,055 and 3,264,089, all incorporated herein. A good system is the Osmocote® system employed by Sierra Chemical Co. This is a heat-cured resin system described in United States Patents 4,657,576 and 3,223,518. The Osmocote® resin system involves a copolymer of dicyclopentadiene and alkyd resin from soy bean oil.

Experimental Results

A number of comparative experiments were carried out using controlled release membrane-coated nutrients and examining their effectiveness in promoting the tissue culture growth of plants. These experiments are provided solely to illustrate modes for carrying out the present invention and the advantages derived therefrom. They are not to be construed as limiting the scope of the invention or as limiting the application of the invention to particular environments or species.

Materials and Methods

1. Culture Media. The basal medium contained Murashige-Skoog's (1962) inorganic salts (M/S salts) and vitamins, 100 mg/l m-inositol, and 30 g/l sucrose. Hormones were added as required by the various plant species for shoot proliferation. The pH was adjusted to 5.7 prior to addition of 0.7% agar.

The media were autoclaved in 1 l bottles at 120°C and 20 psi for 17 minutes and dispensed into various sterile containers in a HEPA-filtered laminar flow hood before gelling. Most of the work was done in plastic containers known in the trade as sundae cups, some in standard 25 x 100 mm petri dishes, some in a plastic containerized systems commercially sold as "sandwich

boxes", and some in Magenta boxes. In all cases the medium volume was kept constant at 60 ml per container.

Plants were inoculated onto medium by insertion with forceps and left to grow for 4-6 weeks in culture rooms kept at 23°C, using a 16 hour photo period and cool white fluorescent lights at 400-800 f.c.

Osmocote® brand osmotic release sustained delivery greenhouse soil fertilizer was used as the sustained release nutrient source. This material, which was used as 1-2 mm prills and as microprills, included nitrogen as ammonium, 9.3%; nitrogen as nitrate, 7.7%; phosphorous expressed as P_2O_5, 6%; and potassium expressed as K_2O, 12% (all by weight). This material had the characteristic of giving a sustained release of these nutrients over a period of up to at least about 120 days.

In addition to the NPK major components, the sustained release material also contained, in some cases, a mixture of other minerals. The added mineral formulation had

1.5% w calcium
1.0% magnesium
0.4% iron
0.1% manganese
0.02% boron
0.05% zinc
0.003% molybdenum
0.05% copper
4.0% sulfur

Typically the controlled release material was about 40% nutrient and 60% inerts and controlled release coating.

When the controlled release material was added it was weighed into test tubes and autoclaved. It was poured onto the surface of the medium and spread evenly. The plants were then inoculated onto the surface covered with prills.

When the controlled release material was added, the initial strength of the medium could be reduced for two reasons: (1) to accommodate any "quick" release from broken or imperfect prills, and (2) to start the plants off at a lower salt level and build it up over time. The initial strength of the M/S salts was reduced from full strength to half strength in initial experiments. Some plants cannot tolerate such low levels and 0.7-0.8 times the typical M/S salts were commonly used in those cases.

2. Conductivity Measurements. Conductivity measurements were used as a quick and reproducible way to monitor the release of nutrients to the growth environment. The conductivity meter used was a Chemtrix 103 instrument with automatic temperature compensation, calibrated daily against KCl standard solutions. It was determined that completely filling the probe tip with agar medium excluding air pockets gave the same reading as measuring the same amount of salts in aqueous solution. The hollow tip of the probe was pushed through the agar to fill it completely, care taken to exclude air or plant debris. Conductivity is temperature dependent so care was taken that the agar was not too warm or too cool during measurement.

End point conductivities taken after 4-6 weeks of plant growth showed plant uptake of mineral salts. Desiccated medium was observed to have a higher conductivity than fresh medium. Often at the end of a culture period the medium had a higher conductivity than fresh medium. Often, the ending conductivity can be quite low showing that plants in tissue culture were able to use up much of the salt in the medium.

The rate of nutrient release from the controlled release material into the tissue culture environment was also monitored with conductivity measurements. The total release point was determined by grinding the prills to a powder or by repeated autoclaving. The calibration of the

actual mass of nutrient or, for that matter, any salt to conductivity is curvilinear and often the meter appears to be more accurate in the lower ranges and less sensitive in the higher ranges.

3. Controlled Release Kinetics. Initially, release of salts from the controlled release material was measured in agar since that was most relevant for interpreting plant growth on agar substrate. It was learned that release in water was virtually identical to that in agar, so thereafter release experiments were done using deionized water because of ease of handling. Figure 1 shows the increase in conductivity observed when controlled release nutrients are released into agar and water and illustrates how, by the practise of the present invention, nutrient is delivered to tissue culture media at a relatively constant rate over at least an 8 week period. The volume was scaled down to fit in 25 x 150 mm test tubes to increase replication.

4. Nutrient Depletion. Samples from the culture base were removed weekly and the conductivity of the medium was measured. The decrease in this conductivity was termed nutrient depletion, referring to the mineral nutrients that are measurable by conductivity. As can be seen in Figure 3, each species observed had its own characteristic nutrient consumption (depletion) rate. The estimated conductivity curves for added controlled release material were calculated from the nutrient depletion curve and release curves. As shown in Figure 4, for each species, with its characteristic depletion rate, there is a particular level of controlled release material which holds nutrient concentration most nearly constant while there are other levels which permit the concentration of nutrients to go up or down with time.

5. Bioassays. Length of subculture was limited to 4 weeks for all species even though a typical production protocol may have been 6 weeks. Controlled release

material was always added just a day or two ahead of planting since release started as soon as the particles of controlled release material are wet. The primary data regarding plant grow out was the biomass and appearance of the plants. Endpoint conductivity was a function of controlled release material release rate and was used to calculate Estimated Uptake (initial amount + amount released - endpoint conductivity). Controlled release material was added to two levels of basal salts in the medium (0.5X M/S and 1X M/S). At the lower level, salts should be more limiting and this should have demonstrated the efficacy of the concept. However, some plants had difficulty adapting to 0.5X M/S so the lower level was increased to 0.7-0.8X M/S instead. The endpoint conductivity showed how controlled release material delayed nutrient depletion and since controlled release material was added at three levels, the appropriate dose could be chosen.

Results and Discussion

Object 1: To Be Able to Get Controlled Release Material in Sterile Form. To use a controlled release material in tissue culture it must be obtained in sterile form, i.e., autoclaved, dry heat sterilized, irradiated, fumigated, or chemically treated. We found that this material can be autoclaved separately from the medium quite satisfactorily. When autoclaved in water, a great deal of the nutrient in the material is released. There is some sticking together of the prills from autoclaving, but it seems to be minor. Moisture from the steam dries out over about a week if the prills are left to stand. An experiment on length of autoclaving time indicates that as little as 10 minutes at 250°F and 20 psi is enough to sterilize small lots of prills and that nonautoclaved prills may harbor bacteria. The prills have also been

successfully disinfected using chlorine bleach solution and alcohol.

Object 2: To Investigate the Release Kinetics. One of the major concerns was whether a controlled release product, "Osmocote®", designed for greenhouse soils would function properly in a tissue culture medium composed of 99% water in a gel matrix. This turned out to be the case.

Controlled release material was added to the surface of water agar (nonsterile but containing 50 mg/l Benlate) at levels of 260, 520, and 1040 mg/60 ml medium. The medium was kept in the culture room at 75-85°F and conductivity measurements were taken weekly for up to 12 weeks. The release rate was linear over time and fairly proportional over the doses of controlled release material tested (Figure 1A).

An identical series of controlled release material doses was added to 60 ml deionized water (DI) instead of agar medium. The rate of release was linear as with water (Figure 1B).

Controlled release material was added to 60 ml DI water containing half strength Murashige-Skoog salts (0.5 M/S) to test whether external salt concentration affected release rate (Figure 2). The release rate was substantially linear and identical to the release rate seen with the agar media or plain water, showing that controlled release rate is relatively independent of external salt or external water potential.

An experiment on incubation temperature showed a dependence of release rate on temperature. It was very slow at 40°F and much faster at 100°F . In fact, there was a 10-20% difference in release rate on the culture shelves depending on proximity to a fluorescent light ballast or an air-conditioning duct. Accordingly, a constant temperature was used in the experiments. Longevity appears to be greater than 8-12 weeks judging

from the release rate experiments and the total salt content available for release.

During the course of release rate experiments, it became apparent that the coated nutrient prills were not homogeneous. The phenomenon of uncontrolled "initial release" was traced to uncoated and broken prills (5%) and irregularly shaped, nonspherical prills (24%) in commercial size bags of the product. The uncoated and broken prills released 94% of their contents in 24 hours and nonspherical prills release about 50% of their contents in 24 hours. In fact, most of the rapid release occurred within 5 hours. Select, perfect prills showed less than 2% rapid release. Initial bioassays were performed using unsorted prills. Subsequently, prills were sorted to exclude most of the irregular prills, culling 25-30% of the product in order to reduce the uncontrolled release. For most experiments, prills were weighed into test tubes for autoclaving. When the prills were applied to medium, a spatula was used to dislodge prills stuck in the tube. Some prill breakage does occur, and this would bias the lower-dose treatments more than the higher doses.

Autoclaved prills were a different color from nonautoclaved prills even after sitting on agar or in water for weeks. The coating took on a darker brown color after autoclaving. However, there was no large or consistent effect of autoclaving on release rate in spite of the color difference. In Figure 1, autoclaved prills were designated 'A' and nonautoclaved prills 'NA'.

The controlled release nutrient used had a 17-9-13 formulation. The nutrients come from ammonium nitrate, ammonium phosphates, calcium phosphates, and potassium sulfates. This is a mixture of 60% 15-15-15 and 40% 21-7-14 fertilizers. The microprills of the controlled release materials weigh 2-3 mg each so in a 260 mg treatment there are about 100 prills. This number assures that about 60 prills are 15-15-15 and about 40 prills are 21-7-14. With

larger prills and fewer prills/dose, there is less assurance of the proper mix of the two materials. This problem is not an issue with a single-component type fertilizer. The other reason for microprills is better distribution spread on or mixed throughout the medium.

Dose rates were chosen as follows: Level 1 was set at 260 mg which is equal to the weight of salt in 60 ml of M/S medium (4.3 g/L). This level was referred to as 1X. The other levels were 2X (520 mg) and 4X (1040 mg). The best dose is determined empirically--that which best enhances plant growth.

Object 3. To Study How Plants Deplete Salts in Tissue Culture and to Correlate This with Growth Kinetics

Nutrient depletion curves were generated from 3 crops (Ficus benjamina, Syngonium "White Butterfly", and Boston fern) (Figures 3A, 3B and 3C). There were 4-6 different groups of each of these species grown in rotation such that one group was subcultured each week. Basically, medium conductivity was measured weekly, four dishes with duplicate readings per dish. Coefficients of variation between duplicate readings are commonly 1% and between repetitions of the same treatment about 10%. Culture vigor can be variable depending on explant size, prior history, and extent of endophytic bacteria. Consequently, greater variation can be expected between groups than within the same group measured week to week.

Nevertheless, several principles emerged from these studies. First, NPK nutrient depletion occurred in tissue culture, the degree related to species and cultural practice (Figures 3A, 3B and 3C). Ficus benjamina showed 91% uptake of salts, Syngonium 70%, and Boston fern 36%.

Records of endpoint conductivities show that there is variability even within a given plant type and this generally correlates with visual growth. Endpoint conductivity can give a clue as to the proper length of the subculture interval. It appears that when plants are

growing rapidly, they take up more salts, rather than if more salts are taken up, the plants grow more rapidly. The key, then, is to get plants to grow rapidly and keep them supplied with proper levels of salts.

Second, the mineral uptake appears to increase rapidly during the first two weeks and decrease thereafter. In the case of Ficus, the decrease may have more to do with the total depletion of the medium than a physiological response. Mineral uptake was estimated from the slope of the depletion curve. Towards the end of subculture when salts are no longer being taken up, the slope (uptake) approaches zero. Uptake is maximal when the conductivity of the medium is falling most rapidly. The variation is due to both sampling error and to extraneous factors affecting culture performance. Peak uptake seems to be correlated with the period of shoot multiplication which is followed by shoot elongation and leaf expansion. This may indicate a greater requirement for salts during the first half of the subculture interval. Alternatively, the duration of the multiplication phase might be limited by available salts.

Growth curves were prepared based on scores for height and biomass. Generally, the curves show sigmoidal growth with log and declining phases. In Ficus, shoot elongation and leaf expansion continued after salts had been >90% depleted. And in the others, it seemed growth was curtailed while salts were still available--indicating some other limiting factor.

Object 5. To Obtain Proof of Concept for Use of Controlled Release Nutrients in Tissue Culture

The controlled release nutrient employed does not appear to be phytotoxic. It was added to plant cultures at three levels covering a 4-fold range and no injurious response, except due to salinity was noted. In fact, repeated enhancement of growth with added controlled release material shows that either there was no

phytotoxicity or that any toxic effect was masked by supplying mineral elements. In the release kinetics experiments, and when taking endpoint conductivities, the pH tended to be buffered about 4.8-5.5, perhaps because of the phosphate in the material. Prills in direct contact with the plant (i.e., about the base, or when sprinkled on leaves, in leaf axils) did not produce any acute localized lesions at the point of contact.

The controlled-release nutrient can be used to supply nutrients to match plant needs in tissue culture as shown in Figures 4 and 5. End point conductivity graphs show addition of between 260 and 520 mg of nutrient per 60 ml over the subculture period maintains conductivity near the initial value. (Note: 260 mg of nutrient in this volume equals the nutrient levels achieved with standard levels of Murashige-Skoog salts.) This may be indicative of an optimal dose rate. The uptake of salts by plants changes week by week whereas the release from the controlled release material is linear and constant. Although medium conductivity was not measured weekly in experiments, it can be estimated from the plant uptake curves and the nutrient release curves (Figure 4). This assumes that uptake is unchanged when salts are maintained at higher than normal levels. The resulting salt profile is the sum of plant uptake and nutrient release. During the first week, conductivity of the medium tends to rise a little since the release rate exceeds plant uptake. Then during weeks 2-3 conductivity either rises only slightly or falls (depending on dose) when plant uptake peaks. Finally when the uptake rate declines, the medium conductivity increases gain. The controlled release product is best suited to crops that steadily take up salts with no sharp peaks or that have broader peaks of uptake. Alternatively, a controlled release product can be custom formulated and blended to give second order release kinetics in order to accommodate peak uptake.

Addition of controlled release nutrient to plant tissue cultures usually enhances growth (Figures 6, 7 and 8). When added to media having less than 1X M/S initial strength, growth increases in a dose-dependent manner, at least to the 520 mg level. At 1X M/S initial strength, often just the 260 mg treatment will show enhanced growth. The 1040 mg dose level is almost always phytotoxic on 1X M/S media and only some of the time on <1X M/S media. Surprisingly, some species show little salinity effects with 1040 mg doses (e.g., Syngonium, carnation, potato). For proof of concept, controlled release material was added to 1/2 strength M/S media--presumably where mineral salts would likely be limiting plant growth from the start of culture. Enhancing growth on a 1X M/S medium might be more difficult since mineral salts are plentiful to start with. However, this response was obtained with several species indicating commercial usefulness. These studies show that doses of controlled release nutrient of between 260-520 mg prevent nutrient depletion in a 60 ml sample.

It is significant that growth can be enhanced above that which is commercially obtained. With some crops, adding controlled release material to <1X M/S media will sometimes equal or outdo the commercial controls. This is starting off at less than the usual initial medium strength. These data imply that the controlled release material supplies limiting factor(s), presumably mineral elements, without creating imbalances or introducing toxic factors.

Growth enhancement was obtained for most species of plants with only a few exceptions. The response is quite universal probably because mineral nutrients (particularly N and then P, K) are almost always limiting plant growth.

Apart from the quantitative enhancement of growth, there were other effects attributable to

controlled release nutrient addition that were observed and these are listed in Table 1.

Table 1. Range of Response to Addition of Controlled Release Nutrient

Very common
- Greater multiplication
- More (faster) growth in same/shorter period of time
- Inhibition of apical dominance
- Inhibition of rooting during multiplication
- Inhibition of petiole extension/leaf expansion
- Darker green color (especially on <1X M/S initial medium strength)

Suspected
- Greater number of shoots reaching usable size
- Continued growth over a longer period of time
- Increased rooting capacity/survival in the greenhouse
- Inhibition of early senescence/yellowing
- Inhibition of vitrification
- Inhibition of excess callus growth
- Inhibition/promotion of endophytic bacteria

Object 5. To Examine the Effect of Lowering Initial Medium Strength and Building Up Over Time

In the bioassays, controlled release material was added to media at both 1X initial medium strength and <1X initial medium strength. There were two reasons for doing this: 1) to accommodate any "quick" release from broken or imperfect prills and 2) to start the plants off at a lower salt level and build it up over time. When it was determined that prill sorting could eliminate a great deal of the "quick" release, it became more desirable to use sorted prills since the mineral balance of M/S salts would be less altered. One of the early premises was that the M/S formulation was optimized for "batch" cultures and that initial strength of the medium was set higher than optimal in order to sustain growth over a 4-8 week period.

M/S is said to be a "high salt" medium for tissue culture. If so, then newly explanted material might do better on lower initial medium strength provided that nutrients are continuously supplied. Also, newly explanted or small pieces of tissue might be less tolerant of high salt levels whereas older, larger tissue pieces might tolerate higher salt levels (perhaps depending on surface area/ volume). Then again, as indicated above, salt uptake seems to peak during weeks 2-3. Perhaps, peak uptake reflects peak need. Also, M/S may not necessarily have been set at a supraoptimal level; it might be set at a point of diminishing returns. Maybe some plants grow faster at 2-3X M/S but not so much faster as to economically justify using more than 1X M/S.

Another issue using controlled release nutrient occurs when media conductivity builds up above initial levels--particularly to 2 or 3 more time initial medium strength. As was pointed out above, larger plants may be more tolerant of high salt levels. Also the M/S formulation may not have been set at the upper limit of salt tolerance.

The phenomenon of adaptation to different salt levels is not unusual in tissue culture. When some lines of carnations are subcultured onto 1/2 M/S, a percentage of them will have leaves that bleach white, even though the old medium may have been depleted to a level similar to 1/2 M/S. Subsequent new growth from the shoot tip is not bleached. Carnivorous plants (Dionea, Drosera) which are normally grown on 1/4 M/S, turn completely purple for at least 4 weeks when placed on 1/2 or 1X M/S. Some die but many resume green growth from either the main shoot tip or side buds. The purple leaves do not revert to green and sometimes die. These examples illustrate salt adaptation.

When using controlled release material, the salt level is constantly changing so that adaptation can be

continuously occurring, except at subculture. If there is a lag in growth following fresh subculturing, it may be because of re-adaptation to new salt levels following prior depletion in culture. When controlled release builds up medium strength to high levels at the end of subculture, this can be beneficial if there is a peak in uptake during the first 2-3 weeks. With respect to adaptation, the safest course may be to maintain constant medium strength near starting levels. That way, there is a chance of eliminating a lag without creating another problem by adapting the plant to higher salt levels.

Several species of plants (Ficus benjamina, carnation, Boston fern, potato, Syngonium and Spathiphyllum) were placed on 1X, 2X, 3X, and 4X M/S media and grown for 3 weeks. Only the major salts were increased, not the minor elements or micronutrients. Precipitation was evident at 3X and 4X. Growth was noticeably affected in Boston fern and potato, shortened height in carnation, no effect on Syngonium (except decrease in rooting), and slight stimulation at 2-3X in Ficus benjamina and Spathiphyllum. Some of the reduction in growth did not look like acute symptoms and salinity. This indicates that salt 'overruns' from adding excess controlled release material may not necessarily be detrimental.

Differential mineral uptake probably occurs. The macroelements are so called because of the large quantities utilized in plant growth. Micronutrients are required for proper growth but are taken up in small quantities. The controlled release nutrient can supply macro-and minor elements which are likely to depleted in a closed system such as the tissue culture vessel. For this reason, it is not absolutely necessary that the quality or analysis of the fertilizer salts used in controlled release product be the same as in M/S salts. The added materials are being introduced to the system only to sup-

plement an M/S based medium, not to substitute for it. When growth in vitro depletes major salts, it become less important which salts are supplied than whether salts are allowed to become major limiting factors. While it is possible to formulate controlled release material to duplicate the relative proportions of M/S salts, it may be determined that this is not actually necessary or economically justified in some cases.

Object 6. To Study ways of Extending the Subculture Interval.

Besides obtaining faster growth and better growth, another application for controlled release in tissue culture is to lengthen the subculture interval. The rationale for this is that greater multiplication in a fixed time period or over a longer interval would decrease labor costs. Part of the formula involves reduction in tissue inoculation--the less tissue used, the greater the potential multiplication rate at the end.

Ficus benjamina and Boston fern were grown at two densities: 3 vs. 5 per dish, with or without 260 mg of controlled release material added to 1X M/S media. Clump size was held constant. Fresh weight and medium conductivity were determined at weeks 4, 6, and 8.

Both 3 and 5 density Ficus attained the same fresh weight by week 8. Most of the growth at 5 density occurred by the fourth week. Controlled release treated cultures were generally larger than those without treatment. The effect seemed greater at 3 density than at 5 density (opposite of what was expected). The difference observed with added nutrient disappeared by week 8. 'Three-density' clumps outgrew those at 5 density after the fourth week. Conductivity readings showed that the higher density used up medium salts faster than the lower density, as expected. The supply of controlled release nutrient elevated medium conductivity above that normally

present at each time point. However, the 260 mg rate failed to maintain medium strength near the initial value. A 520 mg rate might have been more appropriate for the 5 density treatments. It is likely that light or space had become more limiting at week 8 than mineral supply.

In Boston fern, the data were more variable. The 3 density plants did not seem to catch up with the 5 density plants over 8 weeks and the addition of controlled release nutrient did not seem to have an effect. Growth may have ceased for the 5 density treatments after week 6, whereas 3 density treatments may have continued growth to week 8. Once again, the 5 density treatments used more salts than the 3 density. In this case, 260 mg of controlled release material maintained conductivity near initial medium strength for both densities. Slower growth cultures benefit more from longer sub intervals. At 10 density, Ficus benjamina slowed growth after week 4. But Boston fern did not slow down until week 6.

All three crops studied in this section were grown in the same type of dish. The Boston fern produced about 18 g, Ficus benjamina produced about 30 g, and potato produced about 10 g fresh weight. The variation in carrying capacity is puzzling. But consider the initial weight of the explants: 2-3 g for Boston fern, 3-5 g for Ficus benjamina, and 0.2-0.5 g for potato. This makes about a 6-9 fold increase for Boston fern, a 6-10 fold increase for Ficus benjamina, and a 20-50 fold increase for potato.

The addition of controlled release fertilizer did not seem to increase the carrying capacity of the system. It did appear to promote faster growth in a given period of time, provided other limiting factors were not overriding it. It is possible that as limiting factors are removed, controlled release nutrient will have an even larger effect on plant growth.

As noted, a significant ingredient to longer subculture interval is lower plant density. At lower density, the onset of limiting factors is delayed, permitting controlled release material to enhance the rate of growth. Hence, controlled release material is a factor in obtaining the highest possible multiplication in order to decrease labor and material costs in plant micropropagation.

Technical Feasibility Conclusions

The feasibility of using controlled-release nutrient in plant tissue culture has been demonstrated.

The prills were readily sterilizeable, using a variety of techniques, without altering their properties. The release mechanism was operative in pure water or in gels, such as agar. Release rates were constant irrespective of the presence or absence of external salts. Sorting to remove nonspherical, broken or uncoated prills eliminated most of the early, uncontrolled release which was initially observed.

It has been shown that explants can deplete tissue culture media of salts to less than 10% of the original amount. This depletion is dependent on length of subculture interval and correlates with the amount of tissue growth, i.e., if plants are growing vigorously, they will use more salts.

The number of prills needed to deliver controlled-release fertilizers was not excessive. Application of the proper dose of fertilizer prills needed to maintain conductivity near initial levels, resulted in a light to medium coverage of the media surface with prills. The pH was buffered at about 5. With controlled release material addition, one can approximate chemostasis with regard to NPK nutrition and pH control. The longevity of the coated product can be 70-90 days--enough to cover a 12 week subculture if desired. This has obvious

implications for crops that produce storage organs (such as bulbs, corms, or tubers).

Explant utilization of medium salts is variable. Factors such as species, prior growth history, and initial explant fresh weight can influence the amount of salt depletion. Where depletion is higher, the addition of controlled release material should have greater effect, and where depletion is lower, it may have only a small effect.

There did not seem to be any phytotoxicity related to the prills or their contents. Diffusion did not seem to be a major limiting factor especially since plants take up salts over a period of several weeks.

On medium containing 1/2 M/S nutrient salts, addition of controlled release material increased growth (multiplication) and promoted darker green color. This addition can stimulate growth on full M/S salts, as well. Occasionally, material added to < 1X M/S medium produced more growth than did M/S medium without material indicating that a lower initial medium strength could be beneficial. The stimulation of growth on 1X medium means that NPK salts are limiting to growth. Maintaining relatively constant salt levels prolonged the period of multiplication before shoot elongation occurs. At a given level of cytokinin, there was more multiplication at higher salt levels. There are several other visual qualitative responses to nutrient addition which have been observed, apart from increased growth.

In summary, the data presented strongly support the potential for commercial use of controlled-release nutrients in plant tissue culture. Many applications can be envisioned using controlled-released formulations of plant growth regulators, pesticides, antimicrobials, sucrose and other chemicals.

WHAT IS CLAIMED IS:

1. In a process of plant tissue culture micropropagation in which an isolated living plant tissue is grown artificially in a controlled environment including an aqueous medium comprising an effective growth promoting level of an essential nutrient and in which the tissue consumes nutrient from said aqueous medium, the improvement comprising adding to said medium a controlled release source of said nutrient, said controlled release source capable of releasing said nutrient over a prolonged period and replenishing the nutrient consumed by the growing tissue.

2. The process of claim 1 wherein the controlled release source of said nutrient is a water soluble salt.

3. The process of claim 2 wherein the controlled release source of said water soluble salt nutrient comprises a solid depot of said nutrient substantially surrounded by a wall which controls the rate of release of said nutrient into said aqueous environment.

4. The process of claim 3 wherein the wall is permeable to water and wherein the release of nutrient results from the generation of osmotic pressure within the wall which forces nutrient out into the aqueous environment from within the wall.

5. The process of claim 3 wherein the wall comprises a heat-cured alkyd resin-cyclopentadiene copolymer.

6. The process of claim 2 wherein the water soluble salt is a nitrogen-containing salt.

7. The process of claim 2 wherein the water soluble salt is a potassium-containing salt.

8. The process of claim 2 wherein the water soluble salt is a phosphorous-containing salt.

9. The process of claim 2 wherein the water soluble salt is a micronutrient salt.

10. In a process of plant tissue culture micropropagation in which isolated living plant tissue is grown artificially in a controlled aqueous medium, the improvement comprising adding to said medium a controlled release source of a nutrient essential to the growth of the isolated living plant tissue.

11. The process of claim 10 wherein the aqueous medium is agar.

12. The process of claim 10 wherein the aqueous medium comprises added nutrients beyond that added via the controlled release source.

13. A controlled release nutrient for use in plant tissue culturing comprising a depot of water soluble solid nutrient, said nutrient including at least one macronutrient selected from the group consisting of potassium-containing salts, nitrogen-containing salts and phosphorous-containing salts and at least one micronutrient selected from the group consisting of iron-containing salts, zinc-containing salts and manganese-containing salts.

14. A controlled release nutrient for use in plant tissue culturing comprising a depot of water soluble solid nutrient, said nutrient including at least one micronutrient selected from the Murashige-Skoog micronutrient elements.

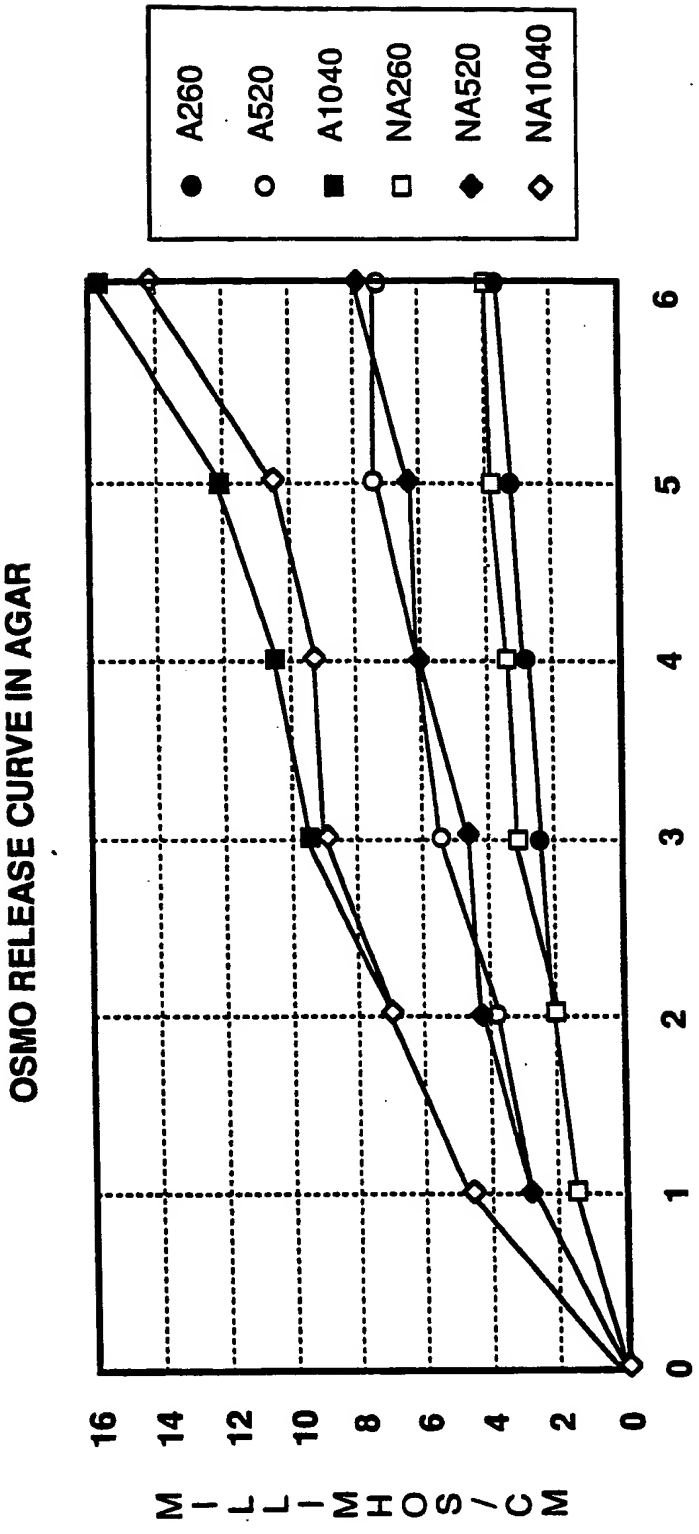
OSMO RELEASE CURVE IN AGAR
A260
A520
A1040
NA260
NA520
NA1040
MILLI-MOS/CM
0
2
4
6
8
10
12
14
16
WEEK
0
1
2
3
4
5
6

FIGURE 1A

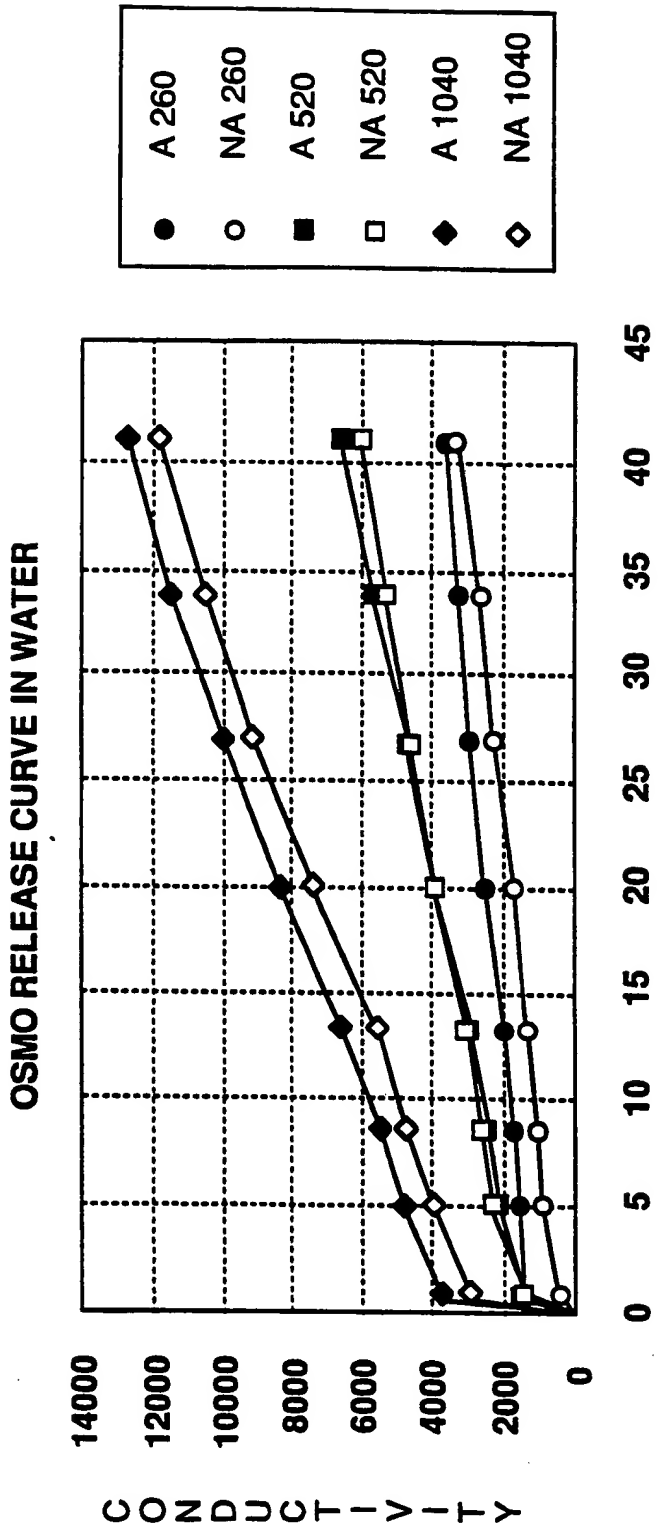
OSMO RELEASE CURVE IN WATER
A 260
NA 260
A 520
NA 520
A 1040
NA 1040
CONDUCTIVITY
14000
12000
10000
8000
6000
4000
2000
0
0
5
10
15
20
25
30
35
40
45
DAY

FIGURE 1B

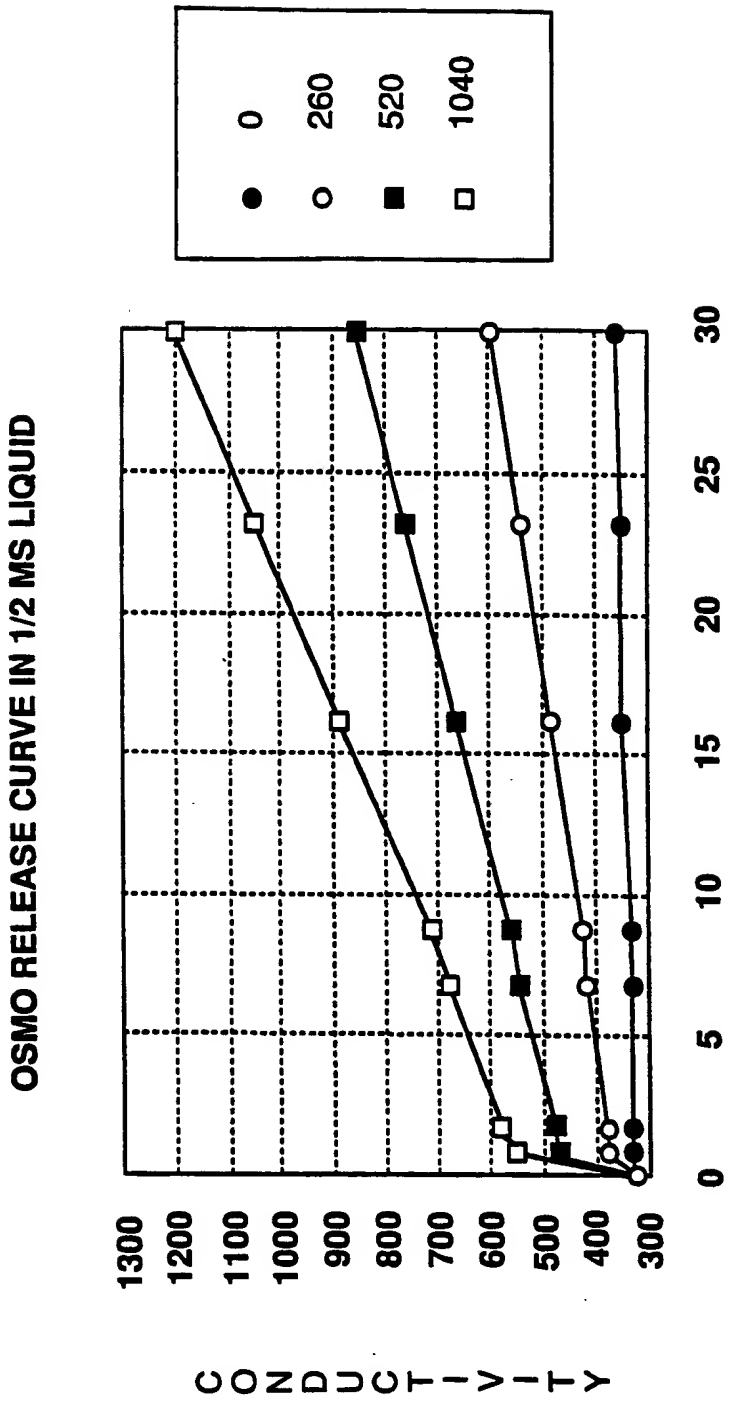
OSMO RELEASE CURVE IN 1/2 MS LIQUID
0
260
520
1040
CONDUCTIVITY
1300
1200
1100
1000
900
800
700
600
500
400
300
0
5
10
15
20
25
30
DAY

FIGURE 2A

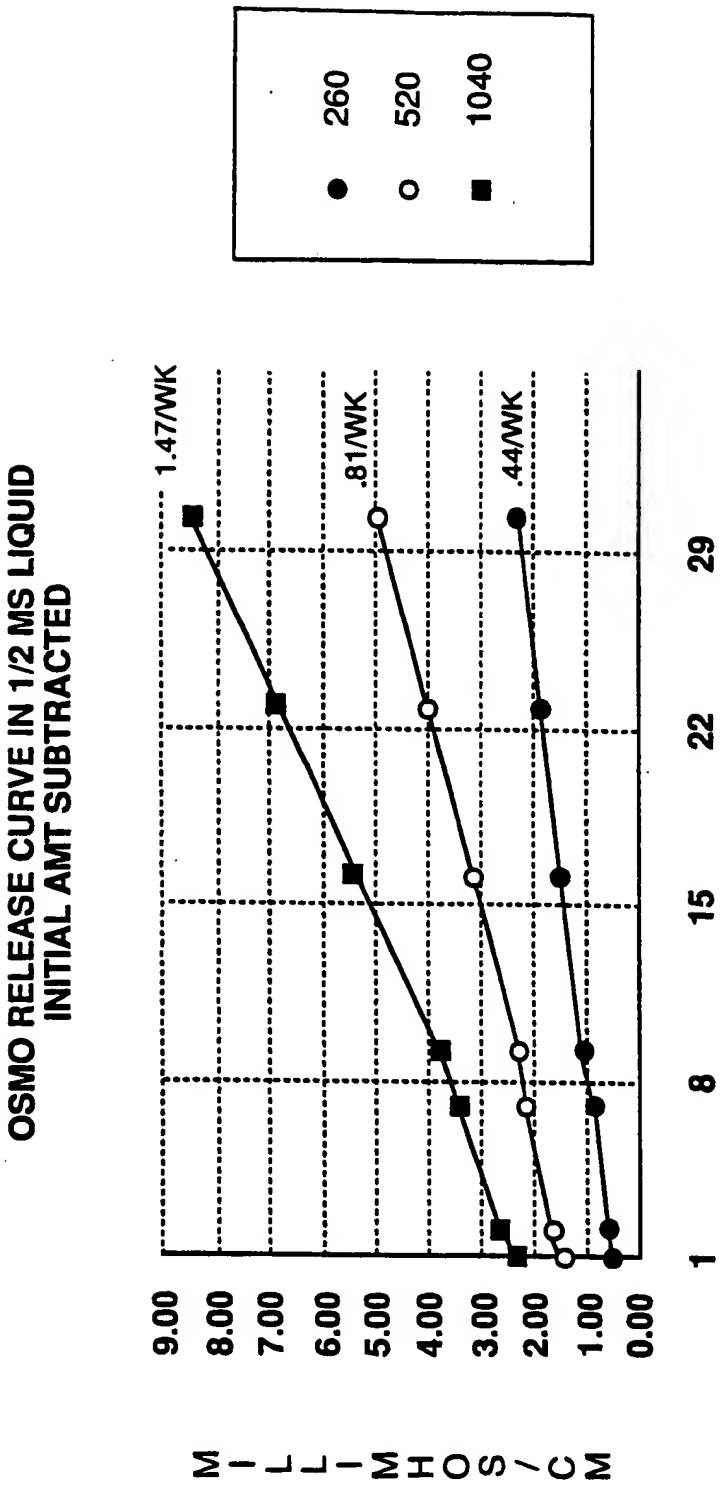
OSMO RELEASE CURVE IN 1/2 MS LIQUID
INITIAL AMT SUBTRACTED
260
520
1040
1.47/WK
.81/WK
.44/WK
MILLIMOS/CM
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
1
8
15
22
29
DAY

FIGURE 2B

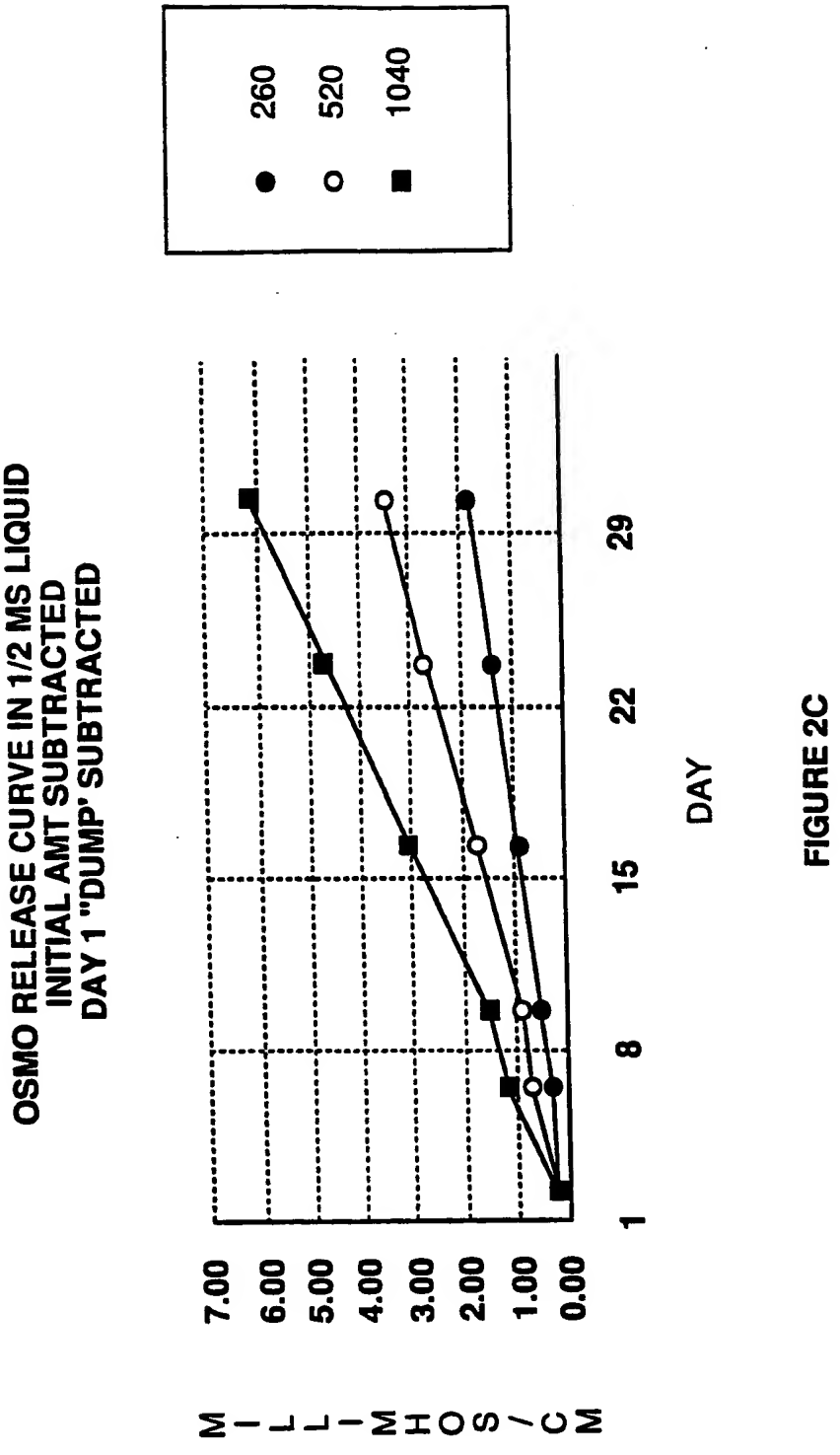
OSMO RELEASE CURVE IN 1/2 MS LIQUID
INITIAL AMT SUBTRACTED
DAY 1 "DUMP" SUBTRACTED
260
520
1040
MILLI-MOS/CM
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
1
8
15
22
29
DAY

FIGURE 2C

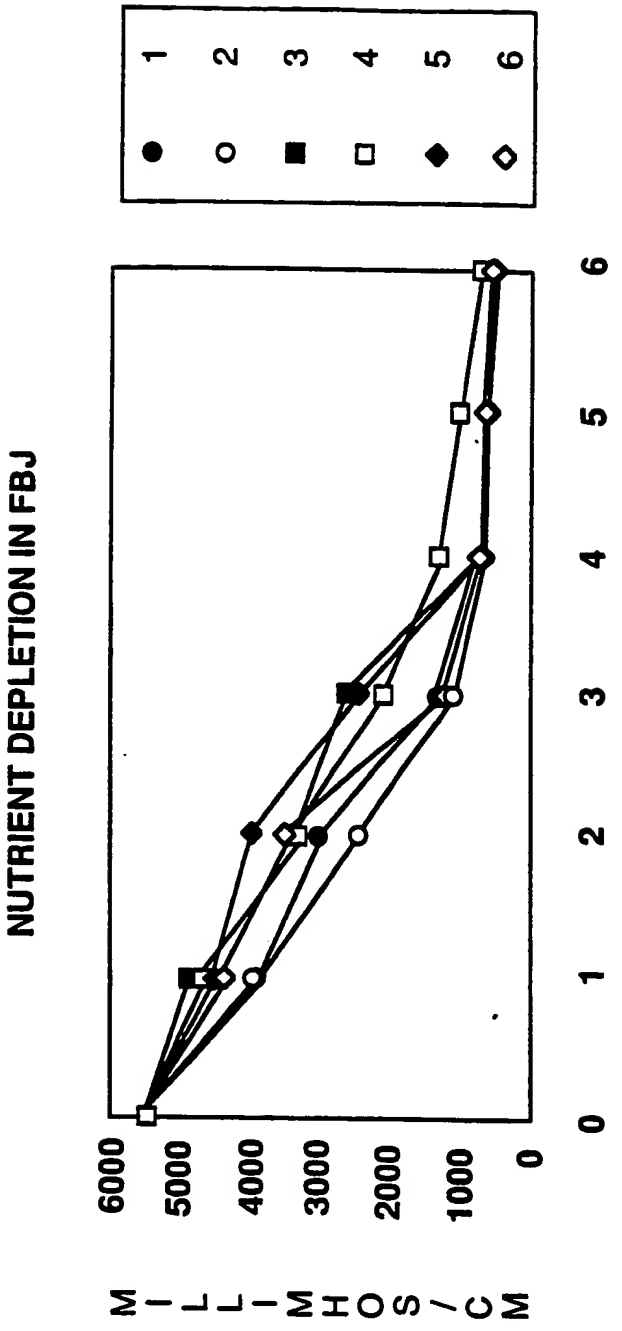
NUTRIENT DEPLETION IN FBJ
1
2
3
4
5
6
MILLIMHOS/CM
6000
5000
4000
3000
2000
1000
0
0
1
2
3
4
5
6
WEEK

FIGURE 3A

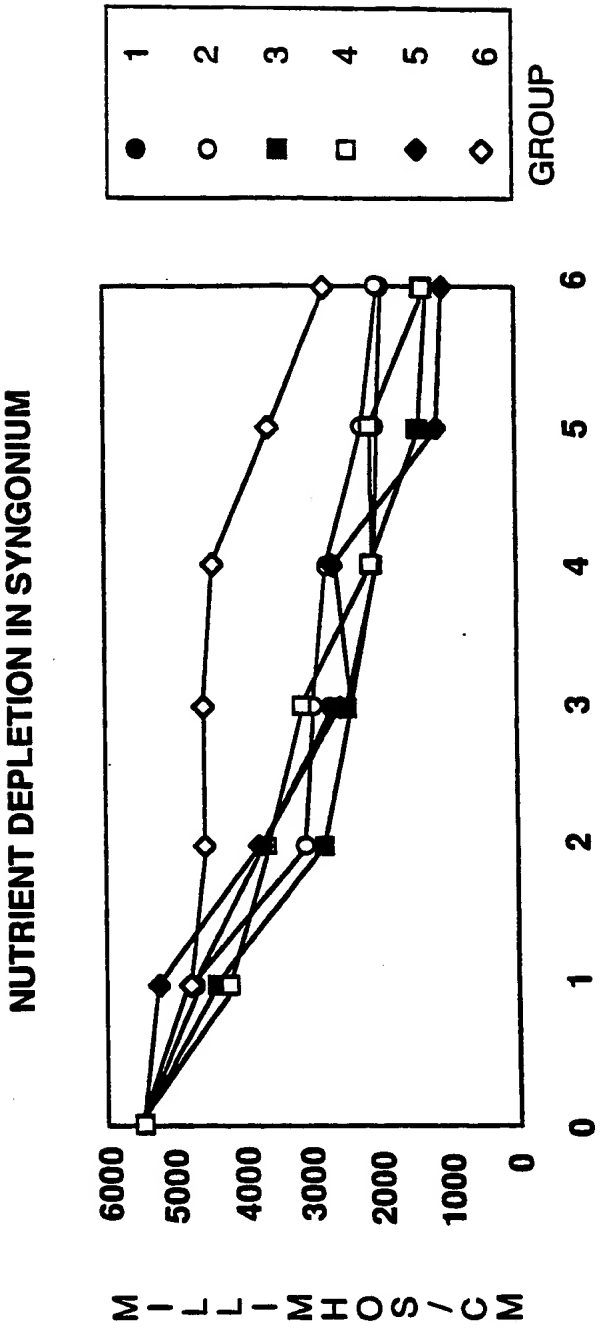
NUTRIENT DEPLETION IN SYNGONIUM
GROUP
1
2
3
4
5
6
MILLIMHOS/CM
6000
5000
4000
3000
2000
1000
0
WEEK
0
1
2
3
4
5
6

FIGURE 3B

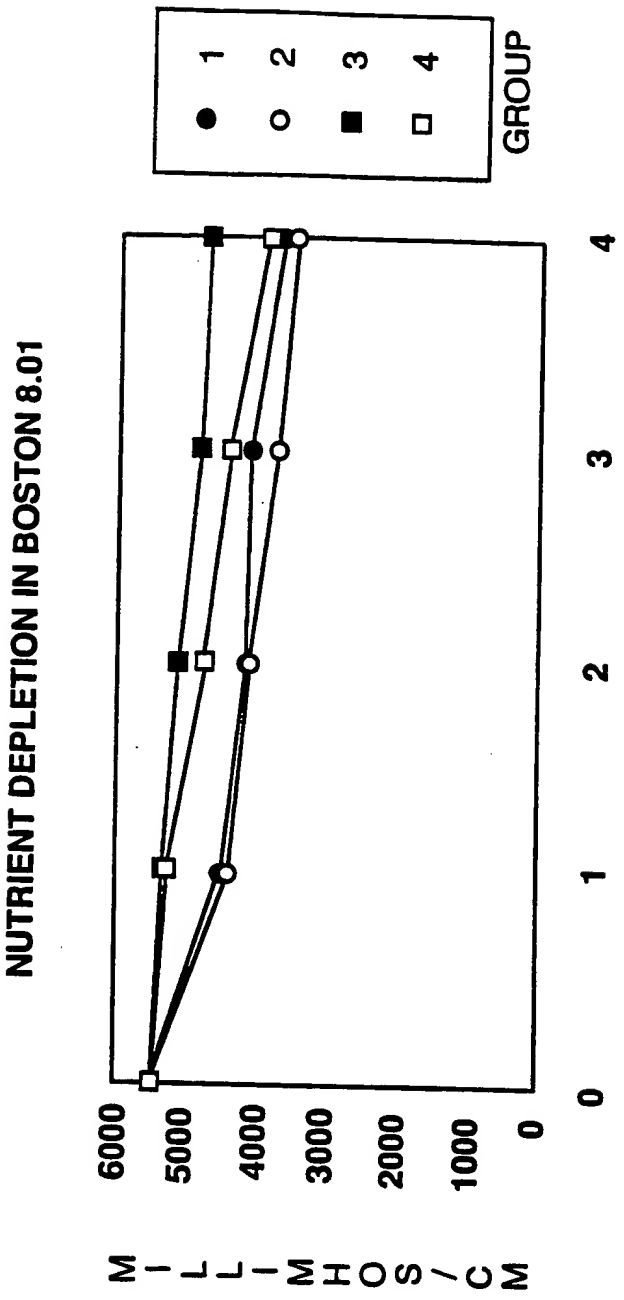
NUTRIENT DEPLETION IN BOSTON 8.01
GROUP
1
2
3
4
MCSOHMFLM
6000
5000
4000
3000
2000
1000
0
0
1
2
3
4
WEEK

FIGURE 3C

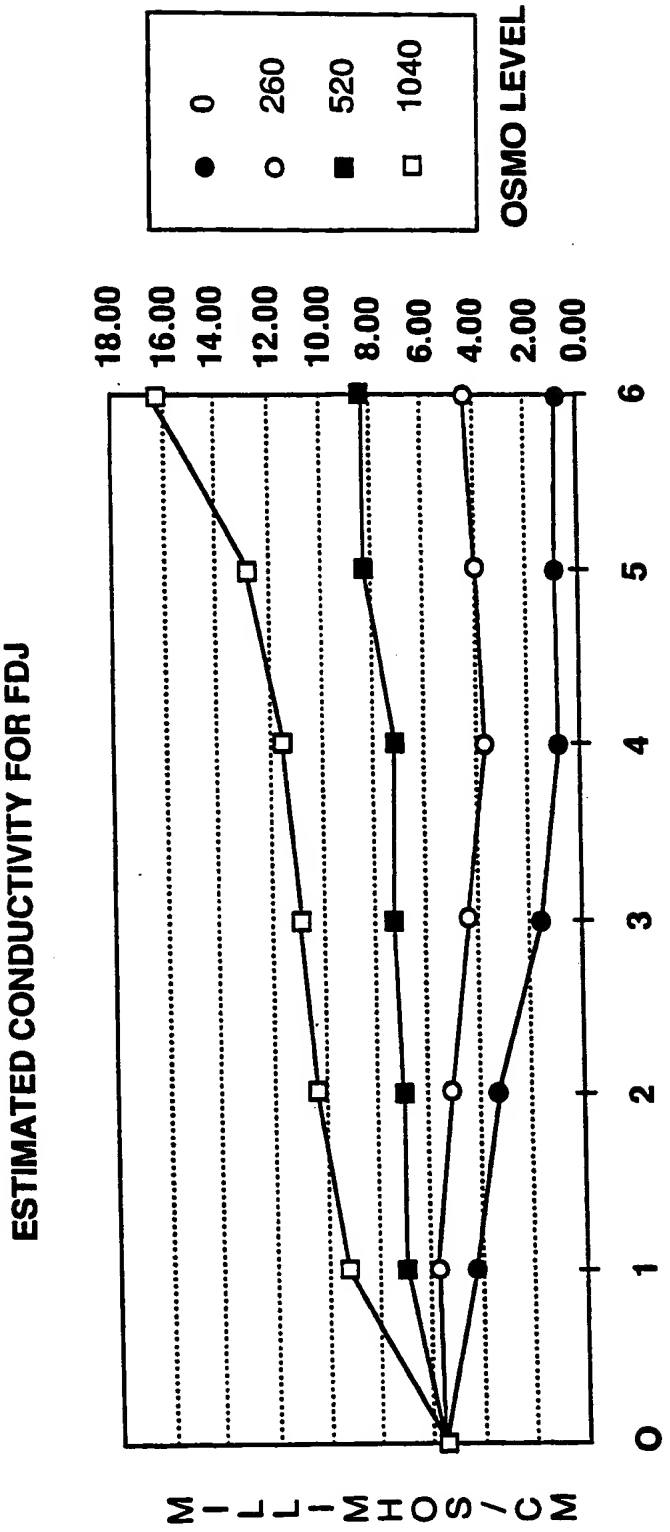
ESTIMATED CONDUCTIVITY FOR FDJ
MILLIMHOS/CM
18.00
16.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
0
1
2
3
4
5
6
WEEK
OSMO LEVEL
0
260
520
1040

FIGURE 4A

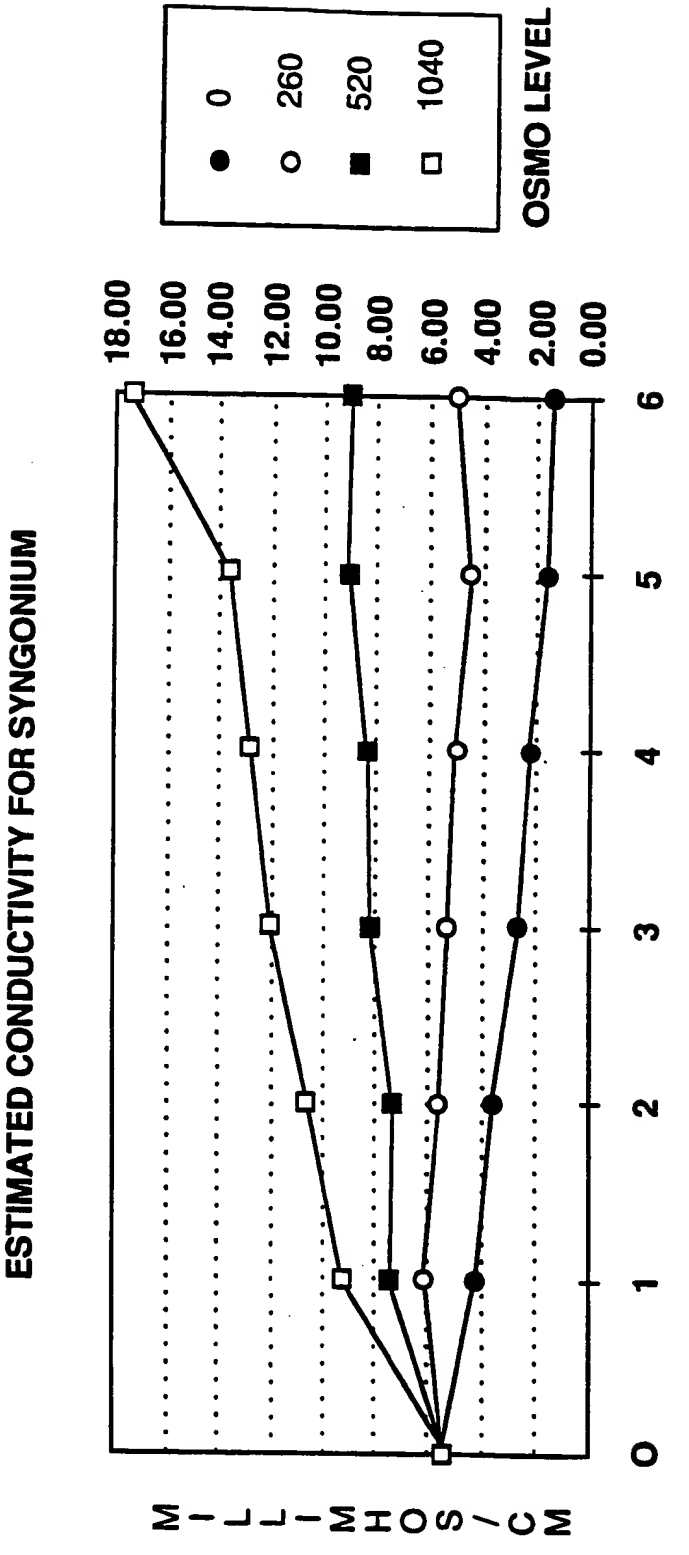
ESTIMATED CONDUCTIVITY FOR SYNGONIUM
MILLIMHOS/CM
18.00
16.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
0
1
2
3
4
5
6
WEEK
OSMO LEVEL
0
260
520
1040

FIGURE 4B

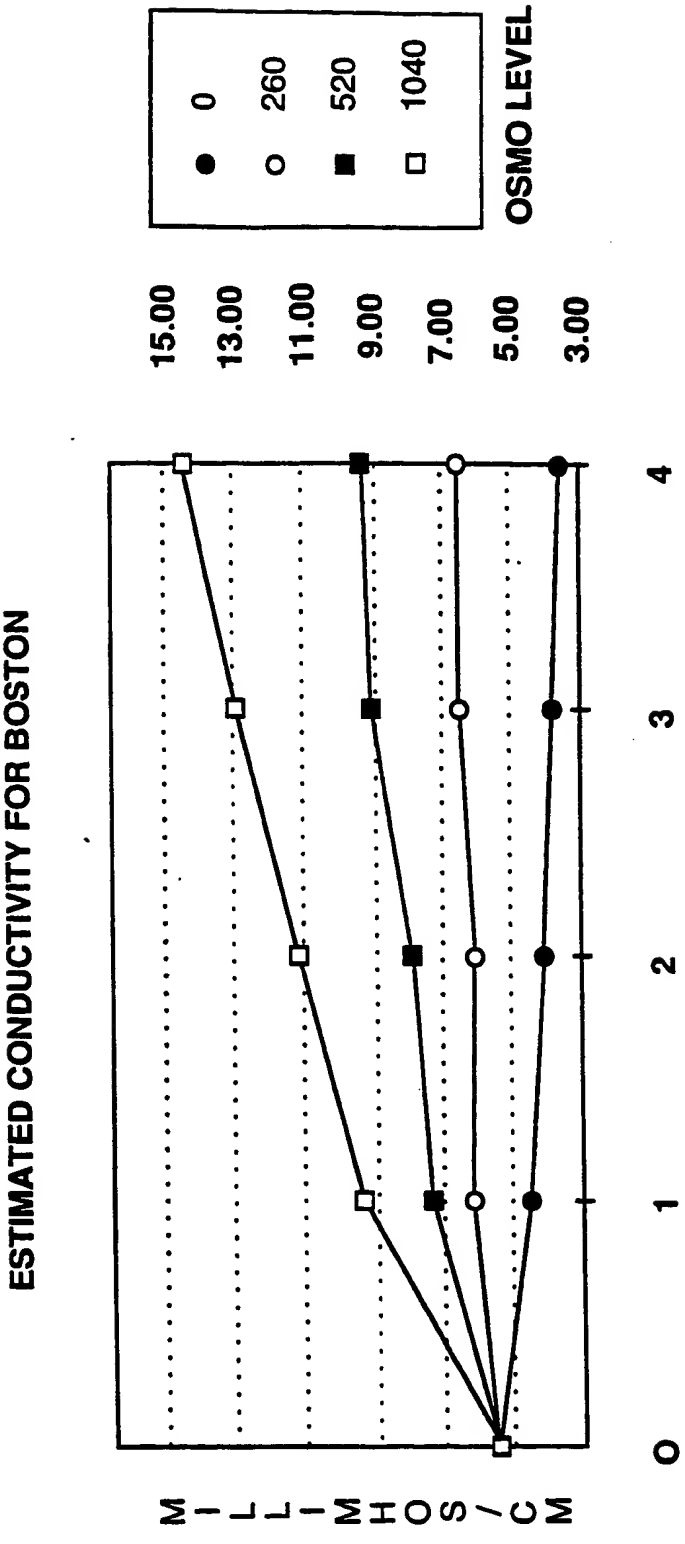
ESTIMATED CONDUCTIVITY FOR BOSTON
MILLIMHOS/CM
15.00
13.00
11.00
9.00
7.00
5.00
3.00
0
260
520
1040
OSMO LEVEL
0
1
2
3
4
WEEK

FIGURE 4C

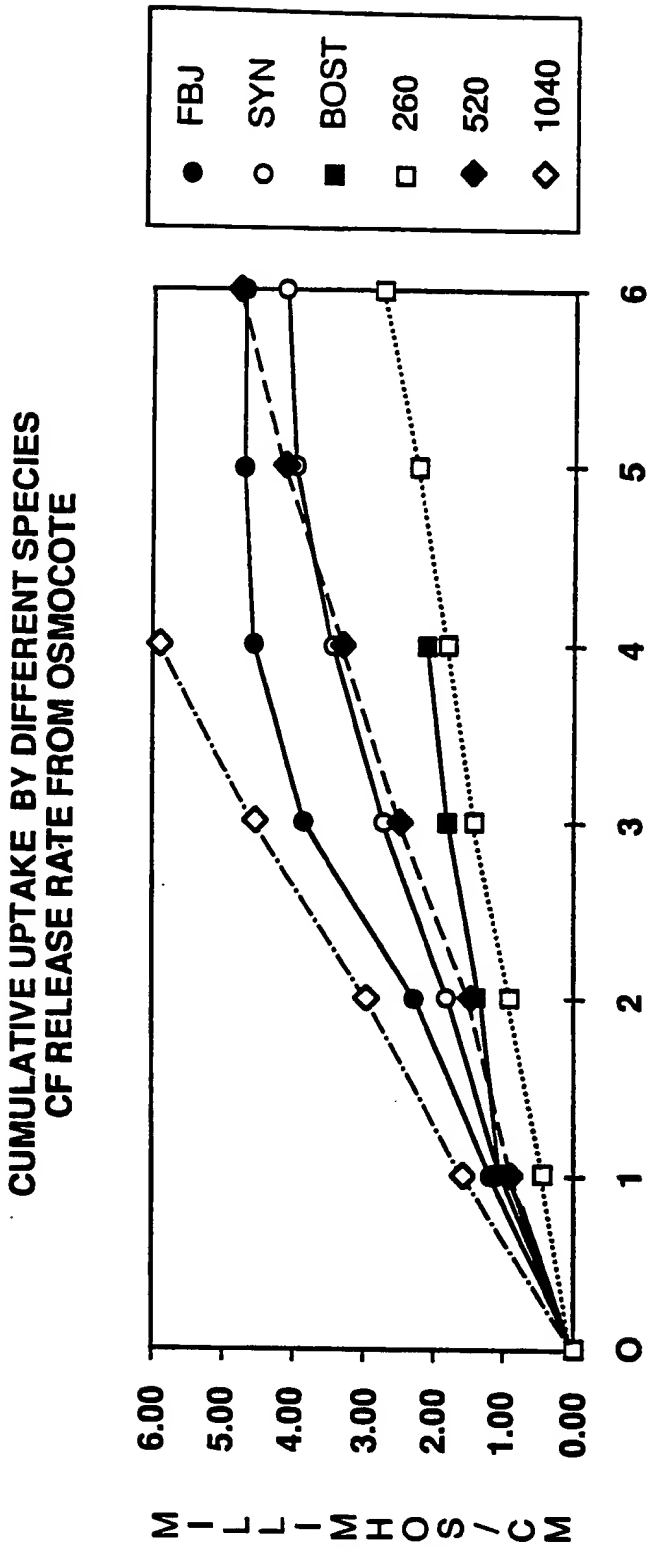
CUMULATIVE UPTAKE BY DIFFERENT SPECIES
CF RELEASE RATE FROM OSMOCOTE
FBJ
SYN
BOST
260
520
1040
MILLIMHOS/CM
6.00
5.00
4.00
3.00
2.00
1.00
0.00
0
1
2
3
4
5
6
WEEK

FIGURE 5

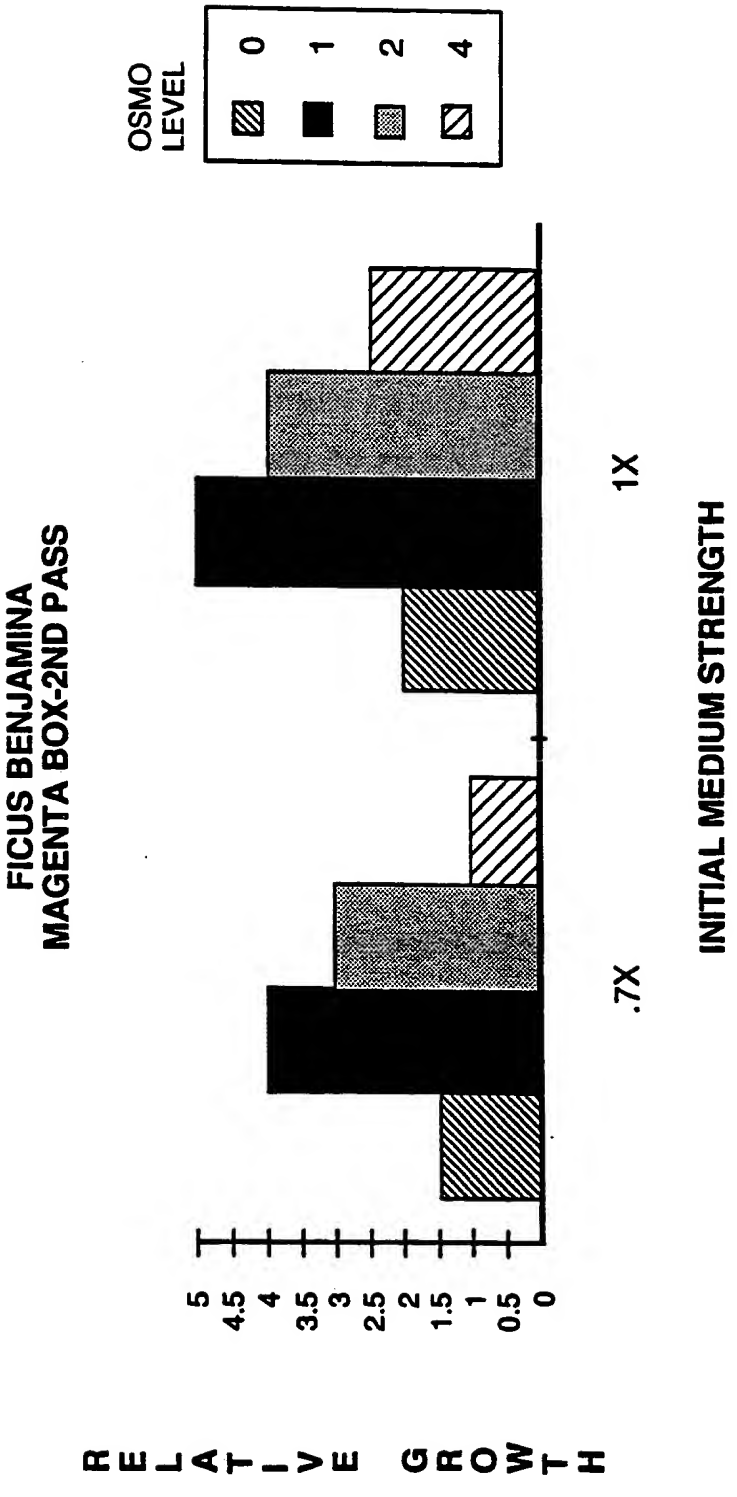
FICUS BENJAMINA
MAGENTA BOX-2ND PASS
OSMO LEVEL
0
1
2
4
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
RELATIVE GROWTH
.7X
1X
INITIAL MEDIUM STRENGTH

FIGURE 6

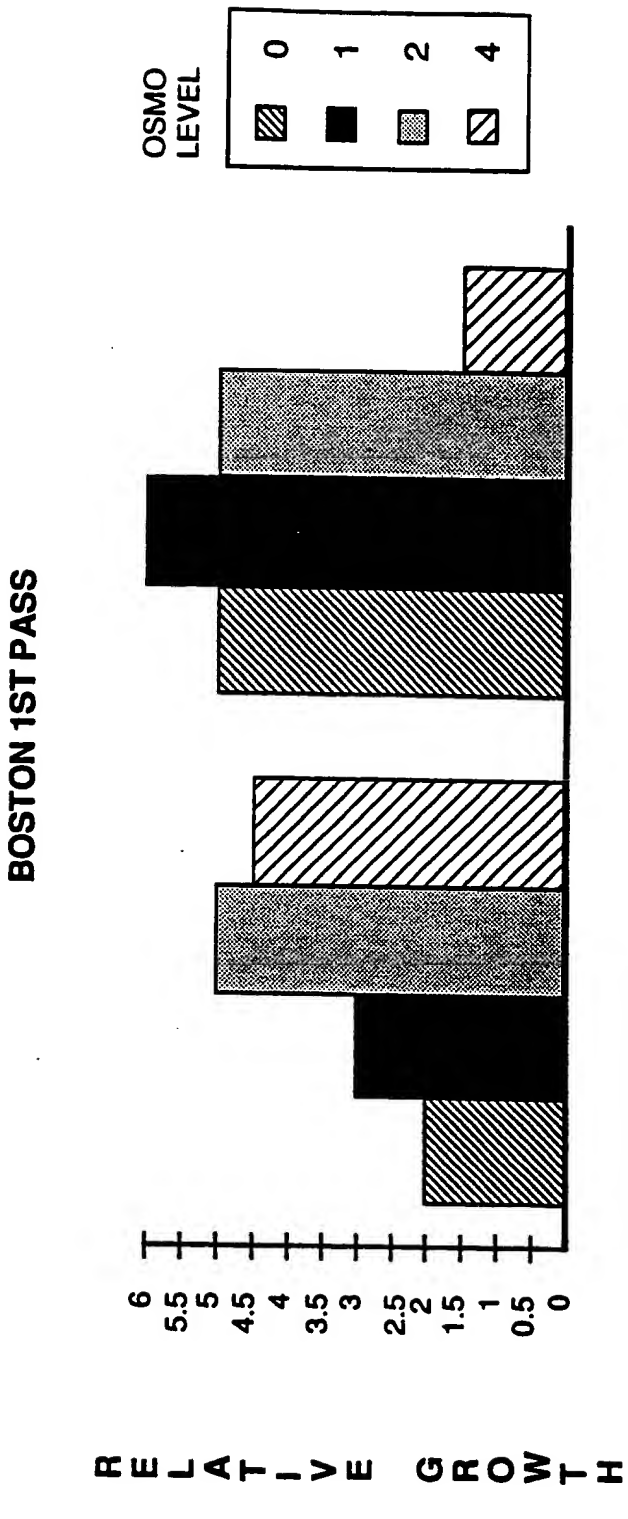
OSMO LEVEL
0
1
2
4
BOSTON 1ST PASS
6
5.5
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
RELATIVE GROWTH
.5X
1X
INITIAL MEDIUM STRENGTH

FIGURE 7

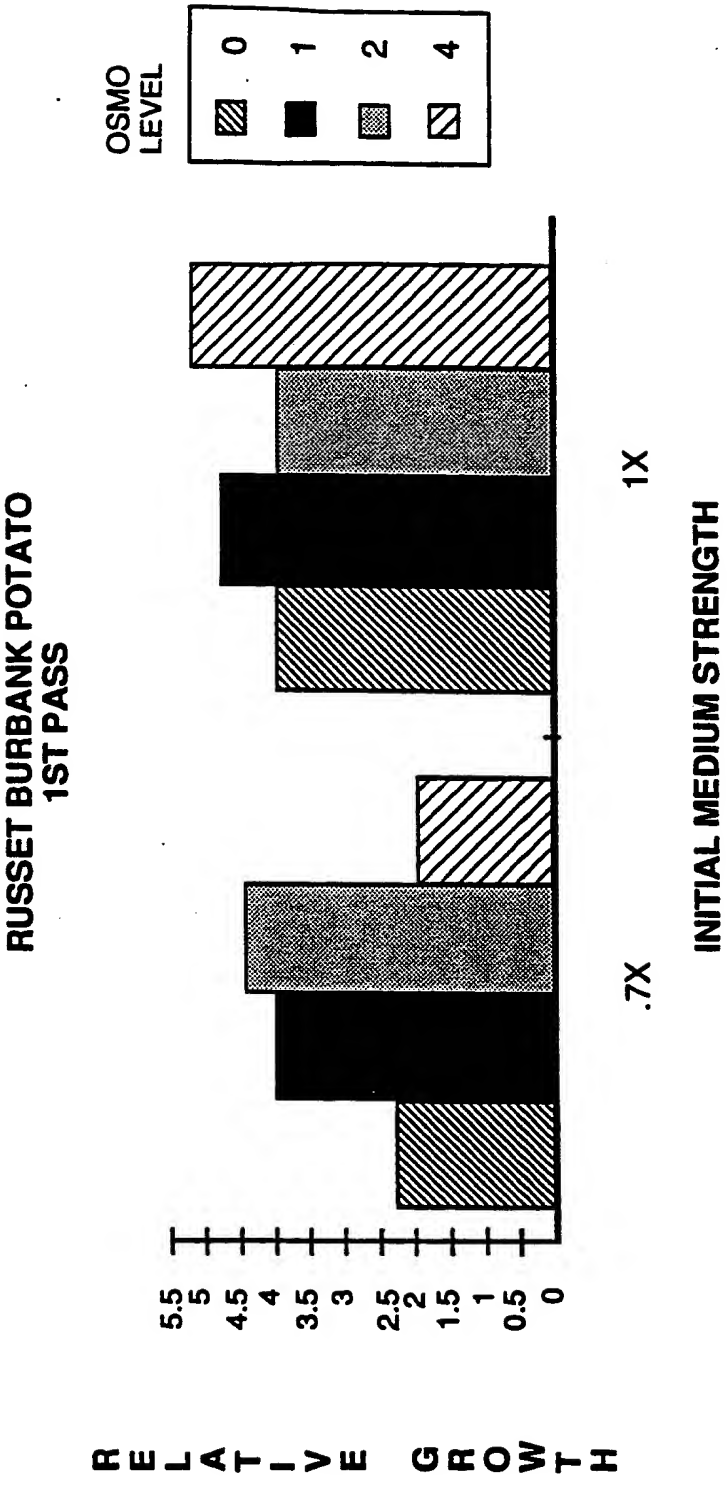
OSMO LEVEL
0
1
2
4
RUSSET BURBANK POTATO
1ST PASS
1X
.7X
INITIAL MEDIUM STRENGTH
RELATIVE GROWTH
5.5
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0

FIGURE 8

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US90/01333

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12N 5/00, 5/02; A01N 25/00; C05G 5/00

U.S. CL.: 435/240.24, 240.241, 240.4, 240.45, 240.46, 240.54; 7/64.11, 64.12

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	435/240.24, 240.241, 240.4, 240.45, 240.46, 240.54; 71/64.11, 64.12

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

DATABASES: Chemical Abstracts Services Online (File Registry and File Biosis); Dialog INFORMATION SERVICES, INC. (BASE AGRI); USPTO AUTOMATED PATENT SYSTEM (File USPAT; 1975-1990)
SEE ATTACHMENT FOR SEARCH TERMS.

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X / Y	US, A, 3,223,518 (HANSEN) 14 December 1965, See the entire document.	13,14 / 1-12
X / Y	US, A, 4,657,576 (LAMBIE) 14 April 1987, See the entire document.	13-14 / 1-12
Y	Communications in Soil Science and Plant Analysis (New York, New York) volume 17(1), Issued 1986, (PILL ET AL.). "Tomato seedling growth in peat and peat-like blocks amended with hydrophilic polymer", pages 45, see the entire document.	1-14
Y	US, A, 4,019,890 (FUJITA ET AL.) 26 April 1977, See the entire document.	13,14
Y	US, A, 4,369,055 (FUJITA ET AL.) 18 January 1983, See the entire document.	13,14
A	US, A, 4,537,860 (TOLBERT, ET AL.), 27 August 1985, See the entire document.	1-12
Y	Hortsuence (St. Joseph, Mo) volume 18(2) Issued April 1983, (POOLE ET AL.), "Establishment and Growth of In vitro-cultured Dieffenbachia", pages 185-187, see especially Experiments 3 and 4.	1-14

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
26 APRIL 1990	25 JUL 1990
International Searching Authority	**Signature of Authorized Officer**
ISA/US	BARBARA M. CHERESKIN

Form PCT/ISA/210 (second sheet) (Rev.11-87)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	Communications in Soil Science and Plant Analysis (New York, New York) Volume 16(8), Issued 1985, (YEAGER ET AL.), "Response of four woody ornamental Species to superphosphate and controlled-release fertilizers", pages 853-863, see the entire document.	1-14
Y	HORTSCIENCE (St. Joseph, Mo) volume 22(5), Issued October 1987, (NEUMAIER ET AL.), "Effect of light and fertilizer Rate and Source on flowering, growth, and quality of hibiscua", pages 902-904, see the entire document.	1-14

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Hortscience (St. Joseph, Mo) volume 20(6), Issued December 1985, (DOUGHTY ET AL.), "Comparison of three Slow-release fertilizers in the production of seedling dwarf date palm", pages 1088-1089, see the entire document.	1-14
Y	Hortscience (St. Joseph, Mo), volume 20(6), Issued December 1985, (DOUGHTY ET AL.), "Evaluation of Liquid and Slow Release Fertilizer Applications in the Production of Dwarf Date Palm", pages 1090-1091, see the entire document.	1-14

Attachment to Form PCT/ISA/210, Part II.

II. FIELDS SEARCHED SEARCH TERMS:

tissue cultur?
Cyclo pentadiene
control release source
Osomocote
Nutricote
Osmocote plus
NPK
NPK1
RN 66455-26-3
Nitrophoska
Suspension culture
Callas
Calli
plantcultur?
control release nutrient